HHS Public Access

Author manuscript

Ann Biomed Eng. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Ann Biomed Eng. 2015 March ; 43(3): 681–696. doi:10.1007/s10439-014-1151-0.

Pre-clinical characterization of tissue engineering constructs for bone and cartilage regeneration

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Abstract

Pre-clinical animal models play a crucial role in the translation of biomedical technologies from the bench top to the bedside. However, there is a need for improved techniques to evaluate implanted biomaterials within the host, including consideration of the care and ethics associated with animal studies, as well as the evaluation of host tissue repair in a clinically relevant manner. This review discusses non-invasive, quantitative, and real-time techniques for evaluating host-materials interactions, quality and rate of neotissue formation, and functional outcomes of implanted biomaterials for bone and cartilage tissue engineering. Specifically, a comparison will be presented for pre-clinical animal models, histological scoring systems, and non-invasive imaging modalities. Additionally, novel technologies to track delivered cells and growth factors will be discussed, including methods to directly correlate their release with tissue growth.

Keywords

Cell tracking; histological scoring; imaging; non-destructive; non-invasive

I. Introduction

In bone and articular cartilage tissue engineering, there are three primary goals in mind for treatment: 1) repair damaged tissue, 2) restore function of damaged articular surface or bone, and 3) fully regenerate the morphological and functional properties of the affected region using the host biological response.[62] Currently, the efficacy of implanted biomaterials to fulfill these goals can only be validated by *in vitro* testing followed by implantation in pre-clinical animal models.[122] The use of animal models provides a more clinically relevant approach as compared to *in vitro* techniques by simulating the dynamic and complex *in vivo* bone and cartilage microenvironment, providing a standardized metric for quantifying biomaterial performance, and allowing clinically-relevant predictions of therapeutic efficacy. A number of animal models have been established and assessed for bone and articular cartilage tissue engineering based on species, defect size and site, surgical

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Conflict of interest: No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

procedure, and implantation period, which has been discussed in other reviews.[22, 59, 61, 85, 104] While animal models are reflective of the human population, translational outcomes are dependent upon the species and tissue type, in which variability and model selection can bias results.

Evaluation of repair outcomes is most commonly limited to discrete time points using histology and mechanical testing, which are invasive, require large animal sample sizes, and provide indirect and qualitative information on the mechanisms and quality of repair by the implanted biomaterial. It would be ideal to develop technologies with equal diagnostic power but minimal surgical invasion, especially with the ability to provide a clear picture of the total defect healing and integration with host tissue. This may include real-time evaluation of bone and articular cartilage repair with the intent to provide diagnostic indicators of disease progression. In general, the evaluation of tissue in a non-invasive, real-time manner would allow a better understanding of repair and growth in a continuous time-scale. Techniques described in this review will include recent progress on the following topics: functional mechanical testing of implants, histological scoring systems, imaging modalities, and growth factor and cell tracking in animal models.

III. *In vivo* characterization of biomaterials for bone and cartilage repair

The development of diagnostic tools for quantifying functional outcomes of therapeutic repair is important for translation from animal models to the clinic. Biomaterial performance *in vivo* is based on metrics that evaluate mechanical, biochemical, and biological changes, including material breakdown, host remodeling, and neotissue growth. Standards for strict evaluation of tissue engineered constructs, particularly in defect models, can be found in Table 1. This section will introduce modalities of functional biomaterial testing *in vivo* using mechanical testing, histological scoring, and imaging, with a specific focus on non-invasive, real-time methods. A summary of select studies and their various modes of evaluation can be found in Table 2.

A. Mechanical testing of bone and cartilage implants

While the mechanical characterization of bone has been well-established for preclinical studies,[3] functional testing of cartilage has yet to be fully characterized due to the inability to fully regenerate the damaged tissue.[63] Furthermore, mechanical tests to assess the shear, tensile and compressive properties of engineered cartilage have been typically destructive. Indentation testing is a type of compressive test that offers a potential technique for *in situ*, non-destructive mechanical analysis of cartilage, and has been used in conjunction with arthroscopy and imaging techniques such as computed tomography, ultrasound (US), magnetic resonance spectroscopy, and x-ray to quantify characteristics such as the dynamic mechanical modulus,[6, 18, 73] cartilage thickness,[6, 18, 73] stiffness,[6, 46, 84, 120] and degeneration.[6, 18] Although in many of these accounts indentation testing was performed on harvested tissue, which does not directly demonstrate *in vivo* diagnostic capabilities for clinical translation, work is continually being done to minimize the invasiveness of this procedure to improve mechanical analysis and understanding of cartilage repair and degeneration. Design considerations for minimizing the invasiveness include the size and geometry of the indenter,[84] since direct contact with the native cartilage may cause abrasion

of the surface.[10] For improvement of diagnostic efficiency, the reproducibility of the measured mechanical forces (of an indentation probe) or output signals (in the case of imaging modalities) is important,[18] especially for user and machine variability. However, fixtures may be designed to reproducibly position the instrument to minimize user variability, which has been demonstrated with an US device used to couple acoustics with mechanical properties.[125] Additionally, measurements may vary depending on the penetration depth of the indenter, so appropriate thresholds should be established before testing.[84] An alternative to contact indentation to consider is water jet indentation, which uses water instead of a physical probe for mechanical measurement, and has been evaluated in tissue phantoms, *ex vivo* plugs, and a rabbit osteoarthritis model with favorable correlation to contact indentation.[80, 127] However, more validation is needed before this can be widely adapted.

Indentation testing can also be improved in combination with other techniques to assess cartilage quality in addition to mechanical measurements. By applying electric fields, electrokinetic measurements can be obtained by detection of current induced-stress gradients that sensitively correlate to changes in the cartilage composition,[101, 133] and ultimately, changes to cartilage health. A promising non-invasive technique that uses this approach and currently being investigated is electroarthrography.[99] The electrochemical properties of cartilage such as fixed charged density can also be obtained by using indentation testing with biphasic and triphasic analyses.[81]

Despite the advantages of indentation testing, one must consider that the mechanical properties of the cartilage are site-dependent, differing in load-bearing or non-load-bearing sites.[46] Models of indentation testing have been introduced to account for cartilage anisotropy,[66] but the validity of these models depends on the quality of the indentation data. Additionally, at the moment, indentation testing provides little information on the mechanical properties of the radial zone of cartilage or the bone-cartilage interface. Therefore, while severe mechanical injury of cartilage may be detectable with this non-destructive method, the difficulty of detecting varying degrees of minor injury continues to be a challenge for both small animal models and diagnosis in the clinic, and has not yet been standardized for functional testing of cartilage. However, the combination of indentation testing, histology, and, particularly imaging, may play an important role in validating the diagnostic capacity of each tool separately.[120]

B. Evaluating biomaterial-host interactions with histological scoring

Histological staining allows for the qualitative evaluation of bone and cartilage tissue microarchitectures with high spatial resolution, morphological fidelity, and specificity with different stains (Table 3). However, when examining histological samples at discrete time points, it is challenging to make quantitative and statistically powered statements regarding tissue development and therapeutic efficacy. Thus, histomorphometric scoring methods provide a means for semi-quantitative analysis of bone and cartilage histology samples.

The nomenclature, metrics, and methods for bone histomorphometry were standardized in 1987,[94] and updated in 2012.[36] Bone histomorphometry focuses on four main metrics: area, length, distance, and number. When adapted for bone tissue engineering, these metrics

provide an assessment of the performance and bone regenerative capacity of a tissue engineering construct in terms of the scaffold degradation, scaffold mineralization and material biocompatibility, and tissue/cell types and number present and volume, surface area, union and thickness of neotissue, respectively.[36] Since there are no standardized global scoring systems for bone, the combination of parameters for histological analysis depend on the defect model, slicing location and direction, and histological stains used.

Unlike bone, the success of tissue engineered cartilage is based primarily on quality of cartilaginous neotissue, making histomorphometric analysis an important measure of outcome. Various osteochondral scoring systems have been established to evaluate the quality of the repair tissue as a whole after implantation of a biomaterial, including: repair tissue thickness and type, cell population and distribution, ECM, and subchondral bone filling, morphology, and type.[82, 91] Commonly used scoring systems include the O'Driscoll score[88] and Bern score,[105] which have been validated and refined in a number of defect models.[14, 57, 97] Other systems such as the Outerbridge score, Oswestry Arthroscopy Score (OAS), and International Cartilage Repair Society (ICRS) scoring of cartilage have been established to classify the severity of the lesion and depth with respect to the subchondral bone, as well as the natural ability of the defect to repair itself.[116] The variety of scoring systems for cartilage evaluation presents questions as to which scoring systems may be validated appropriately for specific applications and will provide useful data for further comparison.[105] While it is difficult to determine if the same scoring system should be valid for scaffolds implanted in different locations of the knee joint (femoral condyle, patellofemoral groove[32]), simplicity is preferred over customization for each case.[105] Before choosing a scoring system, however, it is important to assess its validity in previous experiments and its relevance to the anatomy and physiology of the animal model under investigation.

Ultimately, histomorphometric scoring systems are important measures of outcome for *in vivo* repaired bone and cartilage tissue, but the globally derived scalar value provides only a semi-quantitative and subjective assessment. To improve this, an appropriate statistical analysis, increased quantification of histological samples, and better metrics for repair must be considered. Scoring systems are generally non-parametric and require appropriate statistics to account for non-quantitative data.[58, 71, 95] Although image analysis is not a new concept,[83] advances in computer technology and computing power provide new and automated methods for quantifying tissue growth and scaffold behavior in two-dimensional (2D) histological samples, which warrant further investigation.[51, 111] Additionally, scoring systems using non-invasive imaging such as micro-computed tomography (microCT),[15,53] magnetic resonance imaging (MRI), [85] US,[106] or polarized light microscopy[23] could be implemented to provide quantified measurements to complement histological data. For cartilage histology in particular, new modalities such as CT arthrography and MRI techniques such as delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), T2 mapping, T1rho mapping, ultra-short echo time (uTE), glycosaminoglycan (GAG)-specific chemical exchange saturation transfer (gagCEST), and sodium MRI enable monitoring of disease progression and engineered tissue formation through the quantification of GAGs, proteoglycans (PGs), and collagen content/orientation as imaging measures of outcome.[89]

Lastly, there is a need for improved metrics to assess bone and cartilage repair, including rates of growing tissues, proliferating cells, and decreasing void spaces; to differentiate between heterogeneous tissue samples; and to correlate to other outcome measures.[48] The phenotype of cells, especially macrophages, has become a vital predictor in how the healing process will proceed and should be included in the measure of success as well.[8, 19] Similarly, when employing a disease, infection, or chronic injury in animal models, the use of clinically relevant tests of efficacy such as assessment of nociception, gait, and motor activity should be considered.[42, 87] Increased consensus and standardization in regards to the adaptation of different scoring systems, parameters of measurement, and statistical analysis will enable improved comparison across studies to enhance efficacy of tissue engineered products.

C. Evaluating biomaterial interaction with imaging

When employing imaging modalities to evaluate *in vivo* response to implanted biomaterials, it is important to standardize methods in order to make quantifiable diagnostic conclusions about the biomaterial-host interactions and degree of tissue repair.[15] It is also important to determine healthy baselines depending on the animal, since cartilage thickness and bone structure vary among species.[22, 104] A comparison among several imaging modalities highlights some of the strengths in diagnostic detection of hard and soft tissue disease.[4] It should be noted that while these modalities offer high resolution, 3D images of tissue, it is difficult to evaluate both bone and cartilage simultaneously in a nondestructive manner due to image contrast and interference of soft and hard tissues.[98] Furthermore, visualization and distinction of biomaterials from tissue is still a challenge, and diagnostics of bone and cartilage repair often require validation from histological methods.

i. Magnetic Resonance Imaging (MRI)—MRI is a non-invasive imaging technique that employs strong magnetic fields and radiowaves to excite hydrogen atoms in the body, resulting in image contrast generated from the difference in longitudinal (T1) or transverse (T2) relaxation times of the atoms in different tissues. Due to lack of ionizing radiation or exogenous contrast agents and the advantage of high morphologic resolution, MRI has been employed together with histological scoring systems for bone and cartilage tissue engineering applications to enable semi-quantitative evaluation of bone growth and development, response to mechanical stimulus, structural integrity and fracture, and disease state, [15] as well as monitoring of cartilage disease progression and reconstruction in small animal models and clinically in human patients.[98,50]

Recent advances and validation in MRI techniques have provided new methods to accurately detect and quantify the biochemical composition of tissues. This is particularly promising for osteochondral tissue engineering, where GAGs, PGs, and collagen content/orientation can be used as novel imaging biomarkers in addition to morphologic changes to evaluate cartilage and subchondral bone tissue degeneration and repair, as summarized in previous reviews.[89] Techniques such as T1rho mapping, gagCEST, and sodium MRI enable contrast-free quantification of GAG and PG content through the correlation of T1rho relaxation times with bound water, or sodium signal intensity, to charged macromolecules in the cartilage ECM.[17, 89] Similarly, dGEMRIC measures T1 relaxation times to provide an

indirect measurement and improved resolution of GAG content by utilizing the negative association between an administered negatively charged contrast agent and negatively charged GAG.[50] As a complementary approach, T2 mapping can be used to reproducibly measure structural and content changes in the collagen matrix, which has been validated in a number of clinical studies.[89] Although these techniques have great potential, beyond the general concerns regarding reproducibility, standardization of imaging parameters, and, for dGEMRIC, risks of contrast use, the correlation of these biomarkers to tissue ingrowth in biomaterial scaffolds has yet to be explored in great detail. In this manner, the imaging goal would be to detect an increase or improvement in biomechanical and physiologic function, as well as detect interactions between scaffold degradation and tissue repair. Additional consideration also is needed on how the material will contrast with the tissue and if this will cause interference during imaging.[110]

Regarding the cartilage-bone interface, several improvements have been made employing 3D–spoiled gradient recalled echo imaging with fat suppression (3D–SPGR) and uTE to evaluate the osteochondral junction by using high field strengths and optimized pulse time echo sequences to measure the T1 and T2* (transverse relaxation detected in gradient-echo imaging)[24] relaxation times, respectively.[17, 50] Improvements have also been made in image contrast by taking advantage of fat saturation, water excitation, and changes in molecular charge present in the diseased tissue.[9, 17] Although much has been done with uTE to detect the short T2/T2* relaxation times of the deep radial and calcified zones of cartilage,[89] more investigations are needed to improve MRI techniques for evaluating tissue engineering strategies in animal models.[115] This is especially important for translating preclinical results to human studies, since much higher magnetic fields are often needed to improve the SNR and resolution in small animals, but can be achieved with lower fields for similar diagnostic application in humans. However, animal studies must adhere to ethical safety and care guidelines with respect to possible field thresholds (to minimize tissue heating during imaging)[44] and scan time, where appropriate. Fortunately, researchers have made efforts to standardize the sample preparation, calibration, scan parameters, and data analysis for consistent use of MRI.[15]

ii. Micro-computed tomography (microCT)—MicroCT is another commonly used imaging technique that provides high resolution and non-invasive visualization of bone and hard tissue, especially the microarchitecture, volume, surface area, tissue thickness and spatial distribution of mineralization. [117,123] MicroCT employs x-ray attenuation to create virtual slices of an object that can be reconstructed into an accurate 3D representation. Due to a low voxel size, microCT has arguably higher nominal resolution than MRI,[7] allowing for additional visualization of vasculature and osteocyte morphology,[117] degree of osteoconduction and bone ingrowth,[128] as well as distinct phases of composite biomaterials *in vivo*.[128] Moreover, imaging with microCT enables the characterization of scaffold integrity prior to and after implantation, including biphasic interfaces and tissue mineralization when the biomaterial comprises heterogeneous phases, such as polymer and ceramic components.[109] Techniques have been implemented to approximate scaffold boundaries, cluster size distribution (scaffold and tissue phases), and pore size distribution with microCT.[65] Additionally, microCT can also be used for *in vivo* imaging in small animal

models for longitudinal tracking of bone changes, although there are several concerns over prolonged radiation exposure, imaging duration, and movement artifacts that may be difficult for translation.[16, 4715, 123] A detailed review on microCT evaluation of biomaterials for bone tissue engineering highlights some of the systems used, advances in the technology, and accuracy of 3D reconstructions.[15, 112]

However, microCT for cartilage assessment remains a challenge due to the lack of mineral components in soft tissues for intrinsic contrast.[32] Advances in microCT contrast agents have enabled imaging and quantification of articular cartilage in small animals,[115] and more recently, in large animal models and human patients.[6, 15, 93, 115, 130] Termed contrast enhanced CT (CECT) or equilibrium partitioning of ionic contrast agent microCT (EPIC-microCT), non-invasive imaging of cartilage morphology can be obtained through the inverse correlation of an ionic x-ray absorbing contrast agent with negatively charged PGs and GAGs, and has been demonstrated in humans[118] and large animal models.[6, 12, 76] With appropriate segmentation, even the cartilage-bone interface can be observed.

iii. Ultrasound (US)—US is another diagnostic technique that has been used to non-invasively evaluate integrity of subchondral bone,[54] tissue calcification,[96] fluid content within the joint,[124] synovial integrity,[124] integrity of the cartilage-bone interface,[106] cartilage surface roughness,[126] cartilage thickness,[126] and cartilage disease state.[52] US utilizes externally transmitted sound waves at a frequency of 2–15 MHz to generate structural and functional images in real-time from the mismatch in acoustic impedance in different living tissues.[29] Several US systems and their various modes have been summarized in other reviews.[29, 45] Efforts have been made to employ US as an alternative to biopsies and histological validation of stages of cartilage repair.[53] Despite its ease of use, portability, safety, and non-invasiveness, US has primarily been used in humans and large animal models until recently due to issues with spatial and temporal resolution. Advances in computing and transducer technology have enabled high frequency US systems such as US biomicroscopy, which uses sound waves up to 100 MHz to achieve detailed images of articular cartilage and subchondral bone quality with micron scale resolution.[29, 45] Although minimally invasive, another US technology, the intravascular US (IVUS) device, has been more frequently implemented in preclinical studies since it produces high-frequency images with high resolution through use of a catheter-based probe. By bypassing the superficial layers of skin and muscle, the IVUS can be used in conjunction with arthroscopy in osteochondral defects for improved assessment of cartilage thickness, composition, and structure.[60, 67] However, the technology is limited in evaluating the underlying bone because it is not directly exposed to the surface and rapidly transmits sound. An additional limitation to consider is the possible intra-and inter-user variability that can drastically affect image quality such as the reproducible localization of focal lesions, posing challenges in repeatable quantification of articular cartilage and subchondral bone features.

D. Non-invasive tracking and monitoring

Despite advances in the current methods of *in vivo* analysis, there still remain significant challenges in correlating scaffold behavior and spatiotemporal delivery of stem cells or growth factors directly with tissue formation. Although neotissue growth and functional

restoration are ultimate indicators of efficacy, the development of tissue engineered constructs would benefit from non-invasive monitoring methods to understand of how delivered cells or biomolecules contribute to the regeneration process and compare their kinetics and performance to that of *in vitro*. The following section will provide brief discussion on technologies implemented for non-invasive and longitudinal tracking of stem cell activity, growth factor retention and kinetics, and scaffold degradation *in vivo*.

i. Fluorescent labeling—One of the most common cell tracking techniques is fluorescent labeling, which involves the binding or genetic expression of light-emitting fluorescent dyes or proteins to enable detection of cells using optical methods. Due to their use in pre-clinical oncology research, the breadth and scope of available dyes, reporters, and respective targets are extensive.[100] This is useful in tissue engineering applications since delivered cells can be non-invasively tracked in real-time, allowing for observations regarding engraftment, survival, and tissue induction.[77] Additionally, since the specificity of the fluorescent label can be tailored to different proteins within or outside cells and fluorescence can be coupled with other immunohistochemical probes and *in vitro* studies, mechanistic studies of cells including differentiation, secreted factors, chemotactic effects, and receptor-ligand interactions can be determined, even at the single cell level,[1] to direct cellular migration and future remodeling within the defect site.

Using the red fluorescent dye PKH26, a number of studies have performed non-invasive, real-time tracking of transplanted mesenchymal stem cells (MSC) in a subcutaneous implant model, [131] as well as rabbit[114] and minipig[25] full thickness osteochondral model and sheep joint cavity model, [27] enabling longitudinal data regarding cell localization, viability, and chondrogenic differentiation for cartilage tissue engineering. However, there are a number of challenges for using fluorescence *in vivo*. Fluorescence is prone to photobleaching, leading to a varied duration of expression of reportedly 4–10 weeks,[102, 114, 131, 132] and the resolution and depth of detection is limited by native tissue auto-fluorescence and attenuation, which makes fluorescence detection difficult in large animal models and orthotopic defects. Additionally, the signal is generally not quantifiable, since the fluorescence intensity is affected by the number of cells and their expression efficiency. Near-infrared fluorophores[13, 30, 72] and other fluorescent nanoparticle systems[39, 78] have been developed for improved depth detection and physicochemical stability, but require additional investigation due to detrimental effects to cells.[39, 132]

Through covalent binding of fluorophores directly to the biomaterial backbone, fluorescent labeling can also be used to monitor scaffold degradation kinetics and profile *in vivo*. The degradation of tetramethylrhodamine isothiocyanate (TRITC)-labeled chitosan membranes were successfully monitored in a subcutaneous mouse model for 2 weeks, and the measured fluorescence intensities correlated well with the weight loss of the implants.[31] Membrane fragments were also observed in the neighboring tissue areas, allowing for monitoring of scaffold clearance. Comparisons have been made between *in vitro* and *in vivo* degradation kinetics of hydrolytically degradable fluorescein-tagged poly(ethylene glycol):dextran hydrogels and enzymatically degradable Texas Red-tagged collagen scaffolds.[5] The *in vivo* degradation profiles closely correlated with those of *in vitro*, and differed depending on the location of the implant (subcutaneous, intraperitoneal, or muscle pocket). A number of other

fluorophores[92] as well as imaging methods[70, 134] have also been investigated, demonstrating the importance and challenges of developing a standard non-invasive method for monitoring scaffold degradation *in vivo*.

ii. Bioluminescent imaging—Similar to fluorescent labeling, bioluminescent imaging (BLI) enables the detection of cells through light emission, except that the light is generated through enzymatic cleavage of luciferin substrate by live cells.[33] BLI procedures possess several advantages for tissue engineering applications, including long term expression up to 21 months[113] and enhanced signal-to-noise ratio due to low intrinsic bioluminescence in mammalian tissues.[34] Two of the most common bioluminescent transgenic reporters are firefly luciferase (*Photinus pyralis*) and renilla luciferase (*Renilla reniformis)*. Both reporters have been used for bone and cartilage tissue engineering to monitor and quantify cell migration, distribution, and proliferation in ectopic and orthotopic sites.[34, 79, 90, 113] Through reporter design with specific osteogenic or chondrogenic markers and dual bioluminescence labeling, cell differentiation through the changes in gene expression patterns can also be elucidated.[11, 119] A detailed discussion of BLI transgenic reporters and potential applications specifically for bone tissue engineering can be found in other reviews.[33]

BLI still suffers from some of the limitations of fluorescence imaging, namely, the lack of penetration into hard tissues, and attenuation from neighboring tissue. Additionally, the bioluminescence intensity can be affected by tissue ingrowth into scaffolds and the scaffold material,[2] and altered by the defect model[11, 34] and method of luciferin administration (intravenous, subcutaneous, or intra-articular).[113] Other considerations include the fact that since BLI requires living cells for light emission, implants post-harvest cannot be imaged with bioluminescence, and the transplanted cell population must be easily passaged without losing phenotype in order to obtain a homogenous bioluminescence expression.

iii. Radiolabeling—Radiolabeling is another tracking technique that involves the detection of decaying radioisotopes via nuclear imaging, scintillation, or gamma counter methods. Although some studies have explored its use for cell tracking[49, 129] or scaffold biocompatibility,[108] radiolabeling has been primarily used for monitoring growth factor kinetics, concentration, and bioactivity. Developments in non-invasive growth factor imaging with radiolabeling have commonly been examined in bone tissue engineering applications with iodine-125 (^{125}I),[35, 68] which has a half-life of 60 days and low energy emissions safe enough for biological tissues.[40] Unlike fluorescence imaging or BLI, radiolabeling can provide quantifiable and more stable signals for *in vivo* monitoring without the need for *ex vivo* examination of implants at multiple time points. The release kinetics of ^{125}I-labeled platelet-derived growth factor loaded in chitosan granules in a rat femur defect was successfully monitored weekly for 8 weeks using a probe-type gamma counter with collimator.[35] Validation with *in vitro* delivery data and radioactivity measurements after harvest at each time point demonstrated close correlation with the non-invasive data in terms of both kinetics and dose. By combining radioactivity measurements with radiographic and nuclear imaging techniques, both anatomical and functional information could be evaluated. It was demonstrated that ^{125}I-labeled bone morphogenetic

protein-2 (BMP-2) in both ectopic and orthotopic sites could be monitored up to 8 weeks using scintillation probes, single photon emission computed tomography (SPECT), and microCT.[69] ^{125}I-BMP-2 loaded within different biomaterial scaffolds was able to localize and promote bone regeneration at the defect site without observed differences in the regenerative performance of the BMP-2. Radiolabeling does have several drawbacks, however, including exposure to radiation, loss of tracer *in vivo*, and inaccurate measurements introduced by different labeling methods, detector position, and implant site. Detection of growth factor release from implanted carriers or into directly neighboring tissues is also limited due to poor resolution. Additionally, radiotracers and the sophisticated equipment required for detection can be costly, limiting their accessibility for widespread use. Despite these issues, the ability to sequentially track and correlate growth factor delivery with tissue regeneration is a powerful tool with potential applications in not just bone, but cartilage tissue engineering, where the controlled, spatiotemporal delivery of multiple growth factors is an increasingly employed strategy.[121]

iv. Magnetic particle labeling—Another well-established method for non-invasive *in vivo* imaging is the use of superparamagnetic iron oxide (SPIO) magnetic microparticles and nanoparticles as contrast agents for MRI.[86] The particles consist of an iron oxide core with a surface coating to prevent aggregation and facilitate either cellular internalization or cell surface attachment.[43] The particles can be detected through losses in signal intensity on T2-weighted images via MRI with high spatial resolution. SPIO particles have been used in both cartilage and bone tissue engineering for cell tracking with detection periods up to 12 weeks without significant alterations to cell viability.[55, 64, 75, 103] However, aggregation of the particles remains a concern[107] and some studies show that SPIO labeling may cause subtle differences in the differentiation capacities of labeled cells, resulting in altered ECM morphology.[41] This suggests that even though SPIO technology has a long history in biomedical applications, its use in tissue engineering applications warrants further investigation regarding safety and cell function.

IV. Conclusion

In the recent years, advances in minimally invasive and non-destructive diagnostic technologies have provided powerful new tools by which bone and cartilage tissue engineering therapies can be comprehensively evaluated *in vivo*. In contrast to traditional histological staining and mechanical testing, non-invasive imaging and mechanical testing techniques such as microCT, MRI, US or indentation testing, novel pre-clinical models, and quantified histomorphometric scoring systems enable a clearer understanding of host-material interactions and functional outcomes of implanted biomaterials on a continuous time-scale. Additionally, real-time *in vivo* monitoring via fluorescence imaging, BLI, or radiolabeling provides methods to directly differentiate *in vitro* and *in vivo* performance, correlate controlled delivery with neotissue formation, and elucidate the underlying mechanisms of cell and scaffold behavior. As these technologies are increasingly implemented and refined, there is a definite need for improved standardization and validation of their accuracy for comparisons across studies and significance in results. With any technique, the ability to visualize tissues and biological features with appropriate

resolution limits use in both small and large animal models. Limitations in penetration depth and localization of biomaterial implants and defect sites and instrument dimensions used for testing should be considered to achieve desired resolution. In order to improve resolution in small animals, strategies have been employed, such as the use of contrast (MRI, microCT, US) or higher magnetic fields (in the case of MRI) to reduce SNR, but these strategies may not be directly translatable to humans due to potentially associated cytotoxicities. Conversely, strategies that have been implemented for small animal models will require validation and optimization in order to successfully visualize host-biomaterial interactions in large animals and humans. In combination with the latest advancements in non-invasive *in vitro* techniques and 3D computational modeling, non-invasive and non-destructive diagnostic technologies offer improved assessment and supported conclusions regarding a tissue engineered construct's efficacy and regenerative capacity in order to accelerate their translation to the clinic.

Acknowledgments

We acknowledge support by the National Institutes of Health (R01 AR048756) and the Armed Forces Institute of Regenerative Medicine II (W81XWH-14-2–0004) for work in the areas of bone and cartilage tissue engineering. J.E.T. acknowledges funding from the National Science Foundation Graduate Research Fellowship Program and the Howard Hughes Medical Institute. T.N.V. acknowledges support from a Ruth L. Kirschstein fellowship from the National Institute of Dental and Craniofacial Research (F31 DE023999).

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List of abbreviations

^{125}I	Iodine-125
2D	Two-dimensional
3D	Three-dimensional
3D–SPGR	Three-dimensional spoiled gradient recalled echo imaging with fat suppression
AB	Alcian blue
BLI	Bioluminescent imaging
BMP(−2,−7)	Bone morphogenetic protein (−2,-7)
BSE	Backscattered electron
CECT	Contrast-enhanced computed tomography
DBM	Demineralized bone matrix
dGEMRIC	Delayed gadolinium enhanced magnetic resonance imaging of cartilage
ECM	Extracellular matrix
EPIC-microCT	Equilibrium partitioning of ionic contrast agent micro-computed tomography
FG	Fast green
eGFP	Enhanced green fluorescent protein
GAG	Glycosaminoglycan
gagCEST	Glycosaminoglycan-specific chemical exchange saturation transfer
GT	Goldner’s trichrome
H&E	Hematoxylin and eosin
HA	Hydroxyapatite
hAMSC	Human adipose tissue-derived mesenchymal stem cells
hBMSC	Human bone marrow stromal cells
hMSC	Human mesenchymal stem cells
ICRS	International Cartilage Repair Society
IHC	Immunohistochemistry
ISO	International Organization for Standardization
IVUS	Intravascular ultrasound
Luc	Luciferase

microCT	Micro-computed tomography
MB	Methylene blue
MP	Microparticle
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MT	Masson's trichrome
NF-κB	Necrotic factor- κB
OAS	Oswestry arthroscopy score
OCT	Optical coherence tomography
PAM	Photoacoustic microscopy
ROI	Region of interest
PCL	Poly(ε-caprolactone)
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene glycol)
PG	Proteoglycan
PGA	Poly(glycolic acid)
PIPAAm	Poly(isopropylacrylamide)
PLA	Poly(lactic acid)
PLGA	Poly(DL-lactic-*co*-glycolic acid)
PLLA	Poly(L-lactic acid)
PPF	Poly(propylene fumarate)
PVDF	Poly(vinylidene difluoride)
RGD	Arginylglycylaspartic acid
rhBMP-2	Human recombinant BMP-2
Saf O	Safranin O
SEM	Scanning electron microscopy
SNR	Signal-to-noise ratio
SPECT	Single photon emission computed tomography
SPIO	Superparamagnetic iron oxide
TB	Toluidine blue
TCP	Tricalcium phosphate
TGF-β1	Transforming growth factor-β1

TRITC	Tetramethylrhodamine isothiocyanate
US	Utrasound
uTE	Ultra-short echo time
VG	van Gieson
VK	von Kossa
WK	Working standard (ASTM)

Table 1

Standards for Testing Implanted Biomaterials for Bone and Cartilage Tissue Engineering

Standard		Title
ASTM 561	F	Practice for Retrieval and Analysis of Medical Devices, and Associate Tissues and Fluids
ASTM 565	F	Practice for Care and Handling of Orthopedic Implants and Instruments
ASTM 981	F	Practice for Assessment of Compatibility of Biomaterials for Surgical Implants With Respect to Effect of Materials on Muscle and Bone
ASTM 1903	F	Practice for Testing the Biological Responses to Particles in vivo
ASTM 1983	F	Standard Practice for Assessment of Compatibility of Absorbable/Resorbable Biomaterials for Implant Applications
ASTM 2150	F	Standard Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products
ASTM 2210	F	Standard Guide for Processing Cells, Tissues, and Organs for Use in Tissue Engineered Medical Products
ASTM 2312	F	Standard Terminology Relating to Tissue Engineered Medical Products
ASTM 2315	F	Standard Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels
ASTM 2451	F	Standard Guide for in vivo Assessment of Implantable Devices Intended to Repair or Regenerate Articular Cartilage
ASTM 2529	F	Standard Guide for in vivo Evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone
ASTM 2603	F	Standard Guide for Interpreting Images of Polymeric Tissue Scaffolds
ASTM 2664	F	Standard Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods
ASTM 2721	F	Standard Guide for Pre-clinical in vivo Evaluation in Critical Size Segmental Bone Defects
ASTM 2884	F	Standard Guide for Pre-clinical in vivo Evaluation of Spinal Fusion
ASTM 2903	F	Standard Guide for Tissue Engineered Medical Products for Reinforcement of Tendon and Ligament Repair
ISO 10993		Biological Evaluation of Medical Devices
WK16591		New Guide for Guide for the in vivo Assessment of Bone Inductive Materials
WK28852		New Guide for Pre-clinical in vivo Evaluation in Critical Size Metaphyseal Bone defects
WK31014		New Test Method for Standard Method using Goat for in vivo Testing Articular Cartilage Repair or Regeneration

Table 2

Summary of *in vivo* evaluations of biomaterials for bone and cartilage repair

Species	Tissue	Evaluation period	Construct	Imaging	Histology/ Histomorphometry	Biomechanical testing	Reference
Mouse	Subcutaneous	4 weeks	Biphasic PLA and HA composite seeded with porcine chondrocytes and BMP-7-infected human gingival fibroblasts	microCT	H&E, Saf O/FG	------	109
Mouse	Subcutaneous	1 month	Gelatin and fluorescent hMSCs	Magnetic resonance microscopy, near infrared imaging, *in vivo* small animal imaging, fluorescent microscopy	H&E, VK	------	30
Mouse	Subcutaneous	0, 4, 7, 10, 14 days	Fluorescent chitosan membranes (TRITC)	Cri Maestro™2 *in vivo* imaging system, fluorescence microscopy	------	------	31
Mouse	Calvarial bone	12 weeks	PEG-RGD and Luc (eGFP)-hAMSCs, hBMSCs	*In vivo* BLI, computerizedaxial tomography imaging	H&E, VK, IHC	------	34
Mouse	Subcutaneous	4 h, 1, 3, 5, 7 days	Genipin-cross-linked gelatin conduit with NF-kB-luc	IVIS Imaging System ® (luciferase activity)	H&E, IHC	------	56
Mouse	Subcutaneous	10 weeks	PGA/PLA and goat chondrocytes or PCL/HA and goat BMSCs	------	H&E, Saf O/FG, TB, GT, VK ICRS scoring	Compression	38
Rat	Femora	4 and 12 weeks	Nanofiber mesh, alginate, rhBMP-2 compared to collagen sponge	X-ray, *in vivo* microCT	H&E, Saf O/FG	Torsion to failure	13
Rat	Trochlear groove	21 days	PDGF-loaded heparin-conjugated fibrin, hMSCs	Maestro *in vivo* imaging, fluorescent imaging, confocal microscopy	H&E, Saf O/FG ICRS scoring	------	78
Rat	Patellar groove	3–4 weeks, up to 21 months	Poly(isopropylacrylamide) and poly(vinylidenedifluoride) with chondrocytes, synovial cells	Bioluminescence, IVIS system	Saf O ICRS scoring	------	113
Rat	Subcutaneous	7 days	RGD-alginate or agarose with GFP/Luc hMSCs	Bioluminescence, microCT, flow cytometry	MT, IHC	------	2
Rat	Femur	8 weeks	Chitosan, TCP, and PDGF (cold and radiolabeled with ^{125}I)	Non-invasive and invasive gamma counter	------	------	35
Rat	Subcutaneous and segmental femora	8 weeks	Gelatin, PLGA, PPF with BMP-2, fluorochrome markers (calcein green and tetracycline)	microCT, scintillation probes (growth factor release), fluorescence microscopy	H&E, MB, GT	------	69
Rat	Subcutaneous	2, 4, 7, 10, 12 weeks	Poly[(glycine ethyl glycinato)-$_1$(phenylphenoxy)$_1$ phosphazene] and PLGA	microCT	H&E, MT	------	37

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Species	Tissue	Evaluation period	Construct	Imaging	Histology/ Histomorphometry	Biomechanical testing	Reference
Rat	Femora	4 and 8 weeks	Collagen sponge compared to polyurethane with PLGA MPs with rhBMP-2	microCT	GT	------	20
Rat	Subcutaneous dorsum	1 hr, 14 and 28 days	Chitosan, nano-HA, collagen	US, CT	------	------	28
Rat	Calvarial bone	12 weeks	PLLA and corraline HA scaffolds with and without platelet-rich plasma and bone marrow mononuclear cells	microCT with scoring	GT, H&E, VK, VG with scoring	Push-out testing	74
Rabbit	Patello femoral groove	3 and 6 months	PLGA/β-TCP, type I collagen, rabbit BMSCs	microCT	H&E, TB, Saf O/FG, IHC ICRS scoring	------	32
Rabbit	Distal femoral end	6 weeks	Injectable bone substitute	3D microCT, SEM	2D SEM histology with BSE imaging	Non-destructive analysis of trabecular bone, micro-indentation, compression	47
Rabbit	Middle of patellar groove	4, 8, 12 weeks	Chitosan and gelatin, HA, plasmid BMP-2 and/or TGF-β1, rabbit MSCs	------	H&E, AB, IHC	------	26
Pig	Medial wall of orbit	3 months	Nonporous PLLA/PLA sheeting, PCL scaffolds	microCT	------	------	65
Pig	Patello femoral groove	1 week	Alginate, PKH26-superficial and middle chondrocytes	Fluorescent microscopy	Antibody staining, H&E, Saf O/FG	Tension, confined compression	25

Table 3

Histological Stains for Evaluation of Bone and Cartilage Tissue

Stain	Tissue/cell/ECM-color		Application	Reference
Alcian Blue (AB)	Acid, mucins/ connective tissue	Blue	Cartilage	26
Goldner's trichrome (GT)	Mineralized bone	Blue	Mineralized vs. non-mineralized tissue	20, 69
	Osteoid	Red		
	Nuclei	Blue/ grey/ purple		
	Cartilage	Purple		
Hematoxylin & Eosin (H&E)	Nuclei	Blue	Cellular organization	21, 69
	Cytoplasm	Pink/ red		
Masson's trichrome (MT)	Muscle	Red	Connective tissue, mineralized vs. non-mineralized tissue	2, 37
	Osteoid	Blue/ green		
	Cytoplasm	Red/pink		
	Nuclei	Black/ brown		
Methylene Blue (MB)	Nuclei	Purple	Cellular organization	69
	Connective tissue	Blue		
	Lipid	Red		
	Cytoplasm	Pink		
Safranin O/FastGreen (Saf O/FG)	Nuclei	Black	Cartilage	13, 78
	Cytoplasm/ protein	Blue/ green		
	GAG, mucin, mast cells	Red/ orange		
Toluidine blue (TB)	Nuclear region	Dark blue	Bone or cartilage	32
	Cytoplasm	Light blue		
	Mast cells	Purple		
Van Gieson (VG)	Nuclei	Blue	Collagen and connective tissue	74
	Collagen	Red		
	Cytoplasm/fibrin	Yellow		
von Kossa (VK)	Phosphate and carbonate deposits	Black/ dark brown	Mineralization	34